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Consommation
et Corporations Canada

Consumer and
Corporate Affairs Canada (21) (A1)

2,037,434

Bureau des brevets

Patent Office

(22)

1991/03/01

Ottawa, Canada
K1A 0C9

(43)

1991/09/04

(52)

150-3

C.R. CL. 150-11

5,012,2/06

B15

(51) INTL.CL.⁵ G01N-033/68; G01N-033/86; G01N-033/52; C12Q-001/56;
C12Q-001/37

(19) (CA) APPLICATION FOR CANADIAN PATENT (12)

(54) Functional Assay for Determining the Protein S Activity

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(30) (DE) P 40 06 634.7 1990/03/03

(57) 15 Claims

Notice: The specification contained herein as filed

Canada

CCA 3254 (10-89) 41

BEHRINGWERKE AKTIENGESELLSCHAFT

HOE 90/B 009 - Ma 819

Dr. Pfe/zi

Abstract

Functional assay for determining the protein S activity

The invention relates to a method for the functional determination of protein S in liquids, in particular in plasma, and to a reagent suitable for this.

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Functional assay for determining the protein S activity

5 The invention relates to a method for the functional determination of protein S in liquids, in particular in plasma, and to a reagent suitable for this.

Protein S is an inhibitor of blood clotting and acts as a cofactor of activated protein C in its proteolytic degradation of the clotting factors Va and VIIIa.

10 An inborn or acquired deficiency in protein S can lead to thromboembolic complications.

15 Protein S is synthesized in the liver (Stern, D. et al. (1986), J. Cell Biol. 102, 1971-1978). The biosynthesis is vitamin K-dependent and the concentration in the blood is therefore reduced on treatment with vitamin K antagonists. The start of the treatment can, in the presence of a protein S deficiency, lead to serious impairment of the health of the patient (Grimaudo, V. et al. (1989) BMJ 298, 233-234).

20 The activity may also be reduced in the case of disseminated intravascular clotting or thromboembolic diseases.

25 It is clear from the abovementioned findings that protein S has a decisive influence on the capability of the protein C/thrombomodulin inhibitor system, and that a reliable diagnostic system is correspondingly required. In plasma, only about 40% of the protein S is present in the free form. The rest of about 60% is in a complex with the C4b-binding protein (Dahlbäck, B. (1981), Proc. Natl. Acad. Sci. 78, 2512-2516).

30 This bound protein S is not available as cofactor for activated protein C and therefore has no anticoagulatory

5 action. An excessive proportion of bound protein S can therefore also be found as a functional deficiency, while the antigen is found to be normal or nearly normal (Comp, P.C. et al. (1986), Blood 67, 504-508; Girolami, S. et al. (1989), Thromb. Haemost. 61, 144-147).

10 On the other hand, the activity can be normal in the case of reduced protein S antigen if only a very small proportion is present in the inactive bound form, as is normally the case in newborns (Schwarz, H.P. et al. (1988), Blood 71, 562-565).

It is known to determine protein S immunologically. However, these methods do not provide any information on the activity of the protein and are not a subject of the invention.

15 Methods for determining the activity of protein S have also been described.

20 DE 3724443 A1 describes a method for determining the activity of protein S. This assay requires relatively complex reagents, such as a synthetic substrate plasma, purified factor Xa and prothrombin, and is inconvenient to carry out due to the incubation times. Also, it is interfered with by, e.g., heparin in the sample.

25 DE 3607559 A1 mentions a functional determination of protein S in combination with protein C. It is necessary to determine a reference value without protein C activator for each sample. The use of an activator of F VII or F II is also claimed but no further evidence is shown. Data on the measurements to be expected or interfering influences for all the test systems are not given.

30 A functional assay is described by Comp, P.C. et al. in J. Clin. Invest. 74, 2082-2088 (1984). Here the prolongation of the clotting time by activated protein C is measured in the 1-step factor Xa assay. This assay is

5 relatively insensitive having a prolongation of only 24 sec for the maximum concentration of 50% which can be measured with this method. It was not possible to measure plasmas from patients under vitamin K antagonist treatment or having liver damage.

10 Bertina et al. (Thromb. Haemost. 1985, 53 (2), 268-272) indicate a possibility of determining functional protein S in human plasma. However, the test cannot be evaluated quantitatively for protein S insofar as there is no possible control of the endogenous factor VIII or factor V in the plasma to be measured and the prolongations depend decisively on these two factors. If these factors are present in the sample in an increased or reduced concentration, findings which are correspondingly reduced or increased result for protein S.

20 Van de Waart et al. (1987, Thromb. Res. 48, 427-437) use a system comprising adsorbed substrate plasma, added prothrombin, activated protein C, phospholipids and calcium chloride. A 100% difference in the protein S content of the sample only cause about 20 seconds of prolongation, i.e. the assay is likewise relatively insensitive.

25 Suzuki K. and Nishioka J. (1988, Thromb. Res. 49, 241-251) describe a further assay system. They use Protac®, an activator for protein C from snake venom. This method is also time-consuming and inconvenient. The sensitivity is very low at a prolongation of 13 sec for a 100% difference in activity.

30 Kobayashi I. et al. (1989, Clin. Chem. 35, 1644-1648) also propose a protein S assay which likewise represents a determination via a modified aPTT determination.

The test methods known hitherto have in common that, in addition to an inconvenient procedure because of the multiplicity of reagents used, they only have a low

sensitivity which is measured by the prolongation of the reaction time in relation to the degree of protein S activity.

5 It was therefore an object of the invention to develop a method and a reagent which allows the determination of the activity of protein S in plasma in both a simple and a reliable, sensitive and specific way.

10 According to the invention, a method for determining protein S by measuring the clotting time of a biological sample is now disclosed, wherein the amount of added activators is adjusted in such a way that the clotting time is prolonged beyond the normal clotting time.

15 Prolonging the clotting time made it possible in a surprisingly simple way to achieve a very great increase in sensitivity which can, moreover, be adapted to the requirements.

20 Methods for determining the clotting time are known per se to those skilled in the art. They can, inter alia, be methods which determine the liberation of thrombin from prothrombin via the formation of a clot or the conversion of a chromogenic substrate. The preferred method in this connection is the chromogenic one, with a very particular preference for the use of a chromogenic thrombin substrate, in particular of Tos-Gly-Pro-Arg-ANBA-IPA.

25 In a preferred embodiment of the method according to the invention, an excess of protein S-deficient plasma is added to the undiluted sample and the clotting time is then determined by admixing a reagent comprising
30 activated protein C, an activator of the exogenous or endogenous path of clotting, phospholipids, Ca^{++} , a chromogenic substrate for thrombin and a heparin neutralizing substance. The activated protein C can also be added to the sample separately a short time before the rest of the reagent.

In a particularly preferred embodiment of the method according to the invention, the undiluted sample is admixed with 4 to 10 times the volume of protein S-deficient plasma. The clotting reaction is started by admixing (5 to 10 times the sample volume of) a reagent comprising activated protein C (1 to 50 pmol/ml), an activator of the clotting system, preferably a thromboplastin or a snake venom protease, phospholipids (5-300 ppm (w/v)), e.g. cephalin, calcium ions (2-10 mmol/l), preferably CaCl_2 , a heparin neutralizing substance, such as e.g. Polybren (0.1-10 $\mu\text{g/ml}$) and a chromogene thrombin substrate, such as e.g. Tos-Gly-Pro-Arg-ANBA-IPA. The time from adding the reagent to reaching a certain extinction (e.g. 0.1) at the absorption optimum (e.g. 405 nm) of the liberated chromophor is measured.

The activator concentration which guarantees a clotting time (without protein S) of at least 50 sec under the given reaction conditions, can in each particular case be determined by simple experiments.

Very particularly preferred methods and reagents are those described in the examples.

The clotting time is, when using a chromogenic substrate, preferably determined by determining the time from adding the reagent to reaching a certain extinction at the absorption optimum of the liberated chromophor. For the evaluation, a calibration curve is advantageously prepared by using dilutions of a pool plasma (e.g. 100%, 75%, 50%, 25%, 12.5%, 10%) in the assay and determining the clotting times.

The biological sample can preferably be plasma of human origin, the use of undiluted samples being particularly preferred in this case.

A suitable protein S-deficient plasma can be obtained by

5 methods known per se to those skilled in the art, e.g. by immunoadsorption. Factor VIII is, if necessary, adjusted by adding purified factor VIII. The factor V can, if necessary, be adjusted by adding a protein S-deficient rabbit plasma which contains large amounts of factor V. The volume ratio of sample to protein S-deficient plasma is preferably 1:4 to 1:10.

The deficient plasma preferably has a factor V content of about 20 to 100%, particularly preferably of 50-80%.

10 Protein C can be purified from plasma by various processes which have been described (e.g. Bajaj S.P. et al. (1983) Preparative Biochemistry 13(3) 191-214) or can be prepared by biotechnological processes. The purified protein C can be activated by means of PROTAC® or thrombin
15 which have been coupled to a support material such as Sepharose^(R) or activated protein C obtained directly by genetic engineering processes (Ehrlich H.J. et al., J. Biol. Chem. 264 (24) 14298-14309) can be employed. The concentration of activated protein C in the assay is
20 advantageously between 1 and 50 pmol/ml.

A protein C activated by a snake venom protease from the venom of Agkistrodon contortrix can preferably be employed.

25 Activators of the clotting system are known per se to those skilled in the art. Activators for the purpose of this invention can also be snake venom proteases and activated factors of the clotting cascade, such as e.g. factor VIIa, factor IXa and factor Xa. Preferably, proteases from the venom of Vipera russellii, sulfatides,
30 ellagic acid, thromboplastins and/or silica particles can be employed (Shimada T. et al. (1985) J. Biochem. 97, 429-439). The optimum concentration of the particular activators can be determined by simple experiments.

Phospholipids are a known class of substances which can

be prepared by methods known per se to those skilled in the art or are commercially available. Concentrations of 5-300 ppm (w/v) in the test mixture are preferred. Ca^{2+} ions can advantageously be generated by adding CaCl_2 .
5 Concentrations of 2-10 mM in the test mixture are preferred.

Heparin-neutralizing substances are a class of compounds known to those skilled in the art, such as e.g. Polybren, protamine chloride, protamine sulfate.

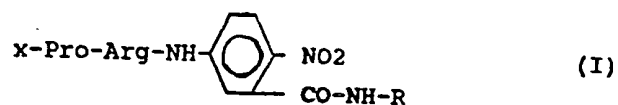
10 The reaction can be carried out at 15-40°C, preferably at 20-40°C, very preferably at 37°C.

It has been found that the protein S present in the plasma can be measured in a sensitive and specific way in the assay described (Table 1). Changes in concentration of factor VIII between 50 and 150%, of the vitamin K-dependent clotting factors, such as prothrombin or protein C (50-150%), except for protein S itself, or the presence of up to 0.4 U/ml heparin have no influence. The factor V content of the sample, as expected, shows a
15 certain influence, since variations in the sample cannot be completely balanced out due to the content in the protein S-deficient plasma and the inhibition of this factor is a measurement of the activity of the protein S.
20

The invention furthermore relates to a reagent for determining protein S by a method according to the invention, the clotting time of a sample without protein S being at least 50 seconds.
25

Preferred reagents are, in this connection, those which contain a protease from the venom of *Vipera russellii* as activator, and those which contain a thromboplastin as
30 activator.

Preferred reagents are also those which contain a chromogenic thrombin substrate of the formula I



where R is C₁₋₅-alkyl or -CH[CH(CH₃)₂]COOCH₃ and
 X is H-D-Phe-, Boc-Gly or tosyl-Gly.

Table 1: Specificity of the assay for the functional determination of protein S

| Changed parameter | Concentration | Protein S activity found |
|-------------------|---------------|--------------------------|
| Control | | 100,0 % |
| Heparin | 0.1 U/ml | 102 % |
| Heparin | 0.2 U/ml | 97 % |
| Heparin | 0.3 U/ml | 109 % |
| Heparin | 0.4 U/ml | 109 % |
| Heparin | 0.5 U/ml | 132 % |
| Heparin | 1.0 U/ml | > 150 % |
| F V | 50 % | 114 % |
| F V | 75 % | 111 % |
| F V | 100 % | 100 % |
| F V | 125 % | 96 % |
| F V | 150 % | 91 % |
| F VIII | 50 % | 106 % |
| F VIII | 75 % | 99 % |
| F VIII | 100 % | 98 % |
| F VIII | 125 % | 103 % |
| F VIII | 150 % | 104 % |
| Protein C | 50 % | 96 % |
| Protein C | 100 % | 100 % |
| Protein C | 150 % | 103 % |

The following examples are intended to illustrate the invention and shall not restrict it in any way.

Example 1

5 Preparation of a ready-to-use reagent based on an aPTT reagent

10 0.5 units of activated protein C and 10 µg polybrene were added to an aPTT reagent (Partochrom®, Behringwerke AG, D-3550 Marburg), comprising phospholipid, sulfatide, Polybren, a chromogenic thrombin substrate (Tos-Gly-Pro-Arg-ANBA-isopropylamide) and Hepes, pH 7.6, and the mixture is heated at 37°C. The reagent is then ready to use.

Example 2

Preparation of a ready-to-use reagent based on a PT reagent

15 0.5 units of activated protein C are added to a PT reagent (Behringwerke AG, D-3550 Marburg) comprising phospholipid, thromboplastin at a low concentration, a chromogenic thrombin substrate (Tos-Gly-Pro-Arg-ANBA-isopropylamide) and Hepes, pH 7.4, and the mixture is
20 heated at 37°C. The reagent is then ready to use.

Example 3

Preparation of a ready-to-use reagent based on an activator from a snake venom

25 40 ng/ml of a snake venom from *Vipera russellii* are added to a buffer comprising phospholipid, a chromogenic thrombin substrate (Tos-Gly-Pro-Arg-ANBA-isopropylamide), a heparin antagonist (Polybren), sodium chloride and Hepes, pH 7.0, and the reaction mixture is heated at 37°C. The reagent is then ready to use.

Example 4

Determination of the protein S content in plasma

5 For the calibration, 100%, 75%, 50%, 25%, 10% and 0% dilutions of a healthy-donor plasma are prepared in phosphate-buffered isotonic saline. The measurement of the standards and the samples is carried out as described below:

10 10 μ l of sample
 50 μ l of protein S-deficient plasma
10 500 μ l of reagent according to a) Example 1
 b) Example 2

are pipetted into a cuvette.

15 When adding the reagent, a clock is started and the extinction at 405 nm is monitored until an increase in the absorption by a certain value (e.g. of 0.1) has been reached. The prolongation of the clotting time beyond the value for 0% protein S is proportional to the concentration of protein S in the sample (Fig. 1).

20 The data for the determination according to the method described in DE 37 24 443 have been taken from German Offenlegungsschrift DE 37 24 443 A1, Figure 1.

Example 5

Determination of the protein S content in plasma when separately adding activated protein C

25 For the calibration, 100%, 75%, 50%, 25%, 10% and 0% dilutions of a healthy-donor plasma are prepared in saline. The measurement of the standards and the samples is carried out as described below:

- 10 μ l of sample
- 50 μ l of protein S-deficient plasma
- 25 μ l of activated protein C
- 500 μ l of reagent according to Example 3

5 are pipetted into a cuvette.

On adding the reagent, a clock is started and either the extinction at 405 nm is monitored until an increase in absorption by a certain value (e.g. of 0.1) has been reached, or the occurrence of clot formation is measured. The prolongation of the clotting time beyond the value for 0% protein S is proportional to the concentration of protein S in the sample (Fig. 2).

10

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE
PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A method for determining protein S by determining
the clotting time in a biological sample, wherein
the amount of added activators is adjusted in such
a way that the clotting time is prolonged beyond the
normal clotting time.
2. The method as claimed in claim 1, wherein an excess
of protein S-deficient plasma is added to the sample
and, after admixing a reagent comprising an
activator of clotting, calcium ions, phospholipids,
activated protein C, an inhibitor of heparin and, if
appropriate, a chromogenic peptide substrate for
thrombin, the time is measured until a defined
increase in extinction at the absorption optimum of
the free chromophor is reached or clotting occurs.
3. The method as claimed in claim 1, wherein the sample
is initially introduced together with an excess of
protein S-deficient plasma and, after separately
adding activated protein C by adding a reagent
comprising an activator of clotting, calcium ions,
phospholipids, an inhibitor of heparin and a chromo-
genic peptide substrate for thrombin, the time is
measured until a defined increase in extinction at
the absorption optimum of the free chromophor is
reached or clotting occurs.
4. The method as claimed in claim 1, wherein the
undiluted sample is admixed with 4 to 10 times the
volume of protein S-deficient plasma, the clotting
reaction is started by admixing (5 to 10 times the
sample volume) of a reagent comprising activated
protein C (1 to 50 pmol/ml), an activator of the
clotting system, preferably a thromboplastin or a
snake venom protease, phospholipids (5-300 ppm
(w/v)), e.g. cephalin, calcium ions (2-10 mmol/l)
preferably CaCl_2 , a heparin-neutralizing substance,

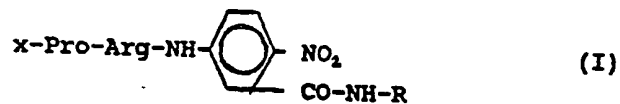
such as e.g. Polybren (0.1-10 μ g/ml), and a chromogenic thrombin substrate, such as e.g. Tos-Gly-Pro-Arg-ANBA-IPA. The time from adding the reagent to reaching a certain extinction (e.g. 0.1) at the absorption optimum (e.g. 405 nm) of the liberated chromophor is measured.

- 5 5. The method as claimed in claim 1, wherein the clotting time is determined with the aid of a chromogenic substrate.
- 10 6. The method as claimed in claim 1, wherein the deficient plasma has a factor V content of 20 to 100%.
- 15 7. The method as claimed in claim 1, wherein the protein C was activated by a snake venom protease from the venom of the snake *Agkistrodon contortrix*.
8. A reagent for the functional determination of protein S by determining the clotting time in a biological sample, wherein the clotting time in the absence of protein S is at least 50 seconds.
- 20 9. The reagent as claimed in claim 8, which contains, inter alia:
activated protein C, an activator of the exogenous or endogenous path of clotting, phospholipids, calcium ions, if appropriate a chromogenic substrate for thrombin, and a heparin-neutralizing substance.
- 25 10. The reagent as claimed in claim 8, wherein the activator is a protease from the venom of a snake or the refractionated snake venom.
- 30 11. The reagent as claimed in claim 8, wherein a thromboplastin is used as activator.
12. The reagent as claimed in claim 8, wherein the

activator is a sulfatide or a mixture of sulfatides.

13. The reagent as claimed in claim 8, wherein the heparin inhibitor is Polybren.

5 14. The reagent as claimed in claim 8, wherein chromogenic thrombin substrate used is a compound of the formula I



where R is C₁₋₅-alkyl or -CH[CH(CH₃)₂]COOCH₃, and
X is H-D-Phe-, Boc-Gly or Tosyl-Gly.

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- 15 -

15. A method for determining protein S as claimed in claim 1 and substantially as described herein.

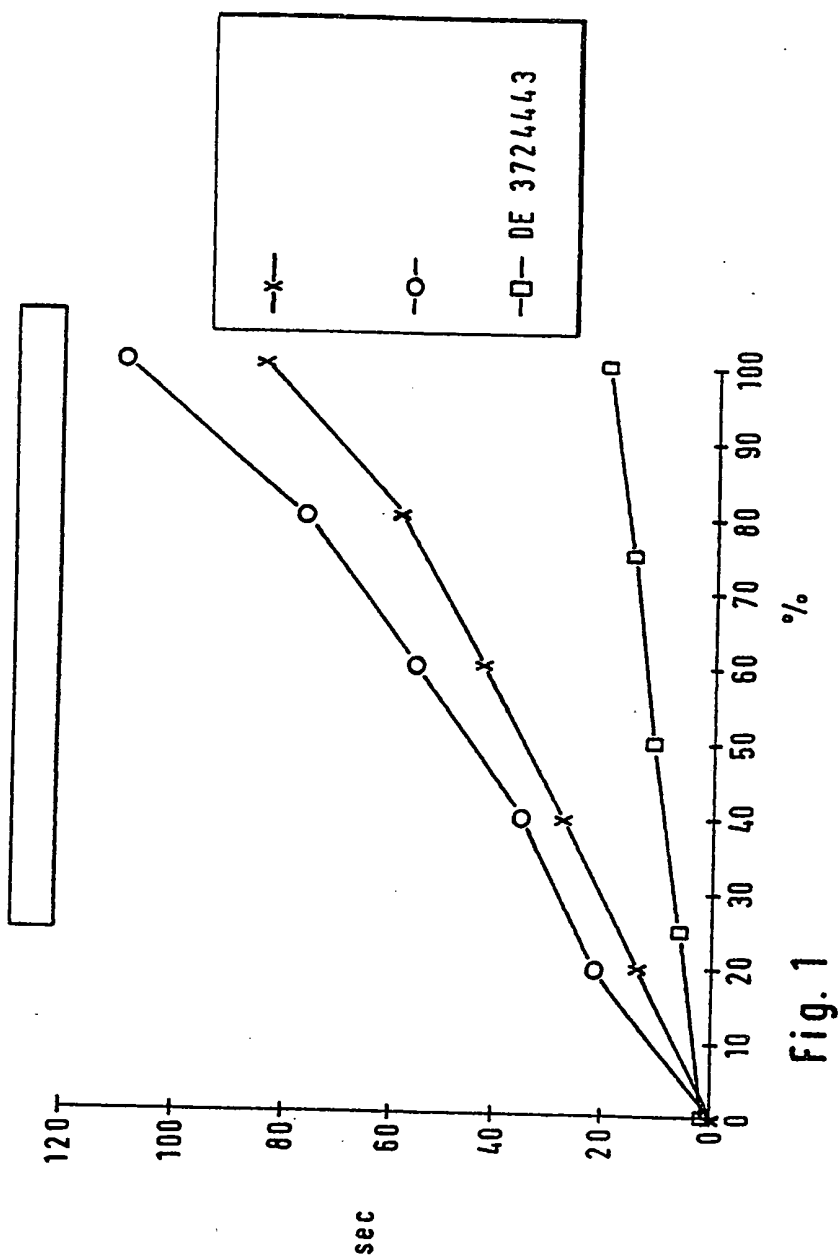


Fig. 1

By: Rogers, Beredkin + Parr